Nonlamellar Phases Induced by the Interaction of Gramicidin S with Lipid Bilayers. A Possible Relationship to Membrane-Disrupting Activity†

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ABSTRACT: The interactions of the cyclic peptide gramicidin S (GS) with a variety of single-component lipid bilayers, and with membrane polar lipid extracts of *Acholeplasma laidlawii* B and *Escherichia coli*, were examined by differential scanning calorimetry (DSC), ^{31}P-nuclear magnetic resonance (NMR) spectroscopy, and X-ray diffraction. The DSC data indicate that the effects of GS on the thermotropic phase behavior of phosphatidylcholine and phosphatidylethanolamine dispersions are compatible with those expected of peptides interacting primarily with the polar headgroup and/or the polar/apolar interfaces of lipid bilayers. These DSC studies also suggest that GS exhibits stronger interactions with the more fluid bilayers. For mixtures of GS with lipids such as phosphatidylcholine, phosphatidylserine, cardiolipin, and sphingomyelin, axially symmetric ^{31}P-NMR powder patterns are observed throughout the entire temperature range examined (0−90 °C), and there is little evidence for significant destabilization of the lipid bilayer with respect to nonlamellar phases. With mixtures of GS with either phosphatidylethanolamine, phosphatidylglycerol, or a nonlamellar phase-forming phosphatidylcholine, axially symmetric ^{31}P-NMR powder patterns are also observed at low temperatures. However, at high temperatures, an isotropic component is observed in their ^{31}P-NMR spectra, and the relative intensity of this component increases significantly with temperature and with GS concentration. Once formed at high temperatures, this isotropic component exhibits a marked cooling hysteresis and in most cases disappears only when the sample is recooled to temperatures well below the lipid hydrocarbon chain-melting phase transition temperature. We also show that GS induces the formation of isotropic components in the ^{31}P-NMR spectra of heterogeneous lipid mixtures such as occur in *A. laidlawii* B and *E. coli* membranes. These observations suggest that GS induces the formation of cubic or other three dimensionally ordered inverted nonlamellar phases when it interacts with some types of lipid bilayers, a suggestion strongly supported by our X-ray diffraction studies. Our results also suggest that the capacity of GS to induce the formation of such phases increases with the intrinsic nonlamellar phase-preferring tendencies of the lipids with which it interacts probably by producing localized increases in membrane monolayer curvature stress. The latter effect could be part of the mechanism through which this peptide exhibits its antimicrobial and hemolytic activities.

The cyclic decapeptide gramicidin S [cyclo(Val-Orn-Leu-D-Phe-Pro)$_2$] was first isolated from *Bacillus brevis* [see Gause and Brazhnikova (1944)] and is one of a family of membrane-active peptides produced by that microorganism [see Izumiya *et al*. (1979)]. Initially, this peptide was shown to inhibit the growth of many species of Gram-positive bacteria, but Gram-negative bacteria appeared to be resistant to its antibiotic effects [see Izumiya *et al*. (1979), Ando *et al*. (1983), and Ono *et al*. (1987), and references cited therein]. Recently, however, the apparent resistance of Gram-negative bacteria to the antibiotic effects of gramicidin S (GS)[1] was shown to be an artifact of the assay procedures used (Kondejewski *et al.*, 1996a). It is now known that if appropriately assayed, GS exhibits a broad spectrum of antibiotic activity against Gram-positive and Gram-negative bacteria and fungi (Kondejewski *et al.*, 1996a,b).

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[1] Abbreviations: GS, gramicidin S; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin; GPDGDG, glycerylphosphoryl diglucosyldiacylglycerol; SpM, sphingomyelin; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; DPEPE, dipalmitelaidoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; TMCL, tetramyristoylcardiolipin; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; T_m, gel/liquid-crystalline phase transition temperature; P_β', lamellar rippled gel phase with tilted hydrocarbon chains; L_β', lamellar gel phase with tilted hydrocarbon chains; L_β, lamellar gel phase with untilted hydrocarbon chains; H_{II}, inverted hexagonal phase; L_α, lamellar liquid-crystalline phase.

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Considerable effort has been directed at defining the relationship between the molecular structure and antibiotic activity of GS and at elucidating the mechanism of its action [for an early review, see Izumiya *et al*. (1979)]. Various structural studies have shown that GS forms an amphipathic, two-stranded, antiparallel β-sheet in which hydrophobic residues (Val and Leu) reside on one face of the molecule and basic residues (Orn) are located on the other face (Hodgkin & Oughton, 1957; Ovchinnikov & Ivanov, 1975; Hull *et al.*, 1978; Izumiya *et al.*, 1979; Rackovsky & Scheraga, 1980). Through the synthesis and characterization of numerous GS analogues and other derivatives [for examples, see Ovchinnikov and Ivanov (1975, 1982), Izumiya *et al.* (1979), Mihara *et al*. (1992), Ando *et al.* (1993, 1995), Katayama *et al*. (1994), and Kondejewski *et al*. (1996b), and references cited therein], it was demonstrated that GS and its active analogues interact very strongly with natural and lipid model membranes and markedly degrade their permeability barrier properties (Finer *et al*., 1969; Pache *et al.*, 1972; Wu *et al*., 1978; Hancock & Wong, 1984; Katsu *et al*., 1986, 1987, 1988, 1989; Portlock *et al*., 1990). Such observations, coupled with the demonstration that the antibiotic activities of GS and its enantiomeric analogue are identical [see Ovchinnikov and Ivanov (1975)], suggest that the antibiotic activity of this peptide is based primarily on its interactions with the lipid rather than the protein components of microbial membranes. Specifically, GS has been proposed to induce the formation of pores in its membrane targets, a mechanistic feature common to many antibiotic peptides [see Heitz *et al.* (1989)]. Unfortunately, GS has the capacity to affect virtually all types of cell membranes and, in fact, exhibits appreciable hemolytic activity (Midez *et al.*, 1989; Lambert & O'Grady, 1992; Katsu *et al.*, 1993). This fact has prevented its therapeutic utilization as an oral or injectable broad-spectrum antibiotic and limited its use to topical applications (Lambert & O'Grady, 1992). The major impetus for continued research on the molecular mechanisms of GS action is to obtain the fundamental knowledge required to develop GS derivatives of comparable or enhanced antibiotic activity but with markedly diminished hemolytic activity.

Although it is generally assumed that the principal target of GS is the membrane lipid bilayer, surprisingly few studies of the interaction of GS with lipid model membrane systems have been performed. Such studies suggest that GS probably interacts primarily with the headgroup and polar/apolar interfacial regions of lipid bilayer model membranes (Datema *et al*., 1986) and that lipid−GS interactions are stronger in the liquid-crystalline than in the gel state (Zidovetzki *et al.*, 1988). It was also demonstrated that the total disruption of liquid-crystalline bilayers occurs at extremely low (<3:1) lipid:peptide ratios (Zidovetzki *et al*., 1988). However, since these studies were performed only on zwitterionic PC-based model membranes, no information is currently available on how the activity of GS and its analogues is influenced by the lipid polar headgroup composition of the membrane with which it interacts. However, interactions between GS and cell membranes have been suggested to be mediated primarily by electrostatic interactions between the basic residues of the peptide and the polar headgroups of anionic lipids which are ubiquitous in such membranes. This suggestion is based on studies showing that the basic amino acid residues of GS are essential for antibiotic activity (Izumiya *et al*., 1979; Ovchinnikov & Ivanov, 1982), that antibacterial activity is very sensitive to the relative positioning of basic residues on the GS molecule (Ovchinnikov & Ivanov, 1975, 1982; Kondejewski *et al.*, 1996b), and that divalent cations impair antibiotic and cytolytic activity, presumably by their interactions with the negatively charged lipids on the surfaces of the cell membrane (Hancock & Wong, 1984; Portlock *et al*., 1990).

It seems likely that interactions between GS and biological membranes should vary markedly with the physical properties, and thus with the lipid composition, of the target membrane. Also, because lipid polar headgroups essentially determine the surface properties of biological membranes, interactions between GS and biological membranes should be particularly sensitive to membrane lipid polar headgroup composition. We have thus begun a broadly based initiative to characterize the nature of the interactions of GS with lipid bilayer model membranes which contain a wide range of polar headgroups. This research is intended to provide basic information on the sensitivity of various membrane lipid classes to the cytolytic action of GS and on how these are affected by variations in the structure of the peptide molecule. Because the lipid compositions of bacterial and eucaryotic cell membranes are quite different, such information could potentially be used in the design of more therapeutically useful GS analogues. We present here the results of DSC, ^{31}P-NMR, and X-ray diffraction studies which show that the interaction of GS with some lipids potentiates the formation of inverted nonlamellar phases, and that the magnitude of this effect is lipid polar headgroup dependent. We also suggest that this tendency may be an integral part of the mechanism underlying the cytolytic activities of GS and its analogues.

MATERIALS AND METHODS

The cyclic peptide GS was obtained from Sigma (St. Louis, MO) and purified by the HPLC methodology described by Kondejewski *et al*. (1996a). DPEPE was synthesized from the corresponding PC by enzyme-catalyzed transphosphatidylation by methods previously used in this laboratory [see Lewis and McElhaney (1993)]. The nonlamellar-forming 1,2-di-(DL-2-butylpalmitoyl)-PC was synthesized and purified as described by Lewis *et al*. (1994). The *Acholeplasma laidlawii* B membrane lipids used were the polar lipid extracts of cells grown in avidin-containing media supplemented with an equimolar mixture of palmitic and oleic acids. The polar lipid extracts were obtained by silicic acid chromatography of the total membrane lipid extracts [see Silvius *et al*. (1980)]. *Escherichia coli* polar lipid extracts were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). It consisted of PE (∼67%), PG (∼23%), and CL (∼10%) (technical information supplied by Avanti Polar Lipids Inc.). The fatty acid composition of the *E. coli* polar lipid sample was palmitate (∼33%), palmitoleate (∼7.3%), 9,10-*cis*-methylenehexadecanoate (∼16.7%), oleate (∼36.9%), and 9,10-*cis*-methyleneoctadecanoate (∼6%) (our analyses). Unless otherwise stated, all other lipid materials used in this study were obtained from Avanti Polar Lipids, Inc. Lipid−peptide mixtures were prepared by codissolving the lipid and peptide in a solvent consisting of 1 part chloroform and 2 parts methanol and subsequent evaporation of the solvent. In those instances where facile handling of the lipid−peptide mixture was required (e.g., the preparation of samples for X-ray diffraction studies), the sample was subsequently redissolved in warm benzene and lyophilized.

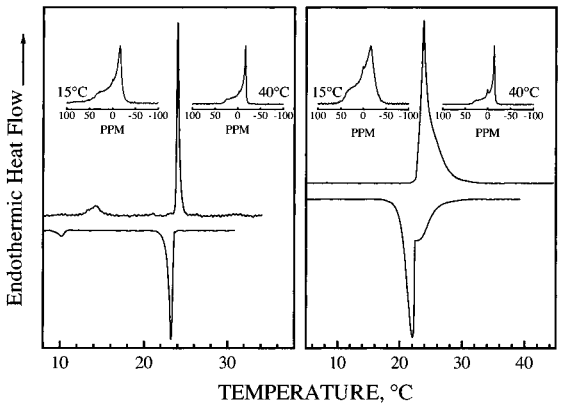

FIGURE 1: Effect of GS on the thermotropic phase behavior of DMPC. The left panel shows high-sensitivity DSC heating and cooling thermograms of DMPC with the insets showing proton-decoupled ^{31}P-NMR spectra acquired in the lamellar gel (15 °C) and liquid-crystalline (40 °C) states. The corresponding data for a DMPC−gramicidin S mixture (25:1) are shown in the right panel.

Unless otherwise stated, samples were hydrated by vigorous vortexing in excess buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, and 1 mM sodium azide, pH 7.4) at temperatures some 10 °C above the T_m of the lipid or lipid−peptide mixture. Subsequently, samples were incubated for 1−2 h at temperatures well below the T_m of the lipid or lipid-peptide mixture (0−4 °C for most samples) prior to initial data acquisition. DSC samples (0.5 mL) contained 2−5 mg of lipid, and thermograms were recorded with a Hart Scientific high-sensitivity heat conduction calorimeter operating at scan rates of 10−15 °C/h. ^{31}P-NMR spectroscopic samples (0.8 mL) contained 10−20 mg of lipid, and spectra were recorded with a Varian Unity 300 spectrometer operating at 121.42 MHz for ^{31}P. Data were acquired and processed using single-pulse excitation techniques as described by Lewis *et al*. (1988). X-ray diffraction measurements were performed with DMPE−gramicidin S mixtures (25:1) using the apparatus previously described in Lewis *et al.* (1989). Specimens for X-ray diffraction were made by hydrating the lipid−peptide mixture with 2−3 times its weight of buffer at temperatures near 60 °C. A typical sample protocol was as follows: The lipid−peptide mixture was placed in a 1.5 mm glass X-ray capillary tube in a water bath, and warm buffer was added with a preheated micropipet. The capillary was then sealed with a 5-min epoxy, and its contents were quickly centrifuged while maintaining a temperature near 60 °C. The sample was then placed in the prewarmed X-ray specimen stage and allowed to equilibrate at 60 °C for 10 h before the X-ray exposure sequence was initiated. The sequence of exposures consisted of a temperature ramp from 60 to 95 °C and then to 40 °C in steps of 5 °C. Each step consisted of 10 min for thermal equilibration and 20 min for X-ray exposures.

RESULTS

DSC thermograms illustrating the effects of GS on the thermotropic phase behavior of large, multilamellar vesicles of DMPC are presented in Figure 1. Unannealed dispersions of the pure lipid exhibit a less energetic pretransition near 14 °C and a more energetic main transition near 24 °C. The pretransition is due to the conversion of the $L_{\beta'}$ to the $P_{\beta'}$ phase and, like all gel/gel phase transitions, exhibits some hysteresis. The main phase transition corresponds to the conversion of the $P_{\beta'}$ to the L_α phase and, like all "simple" hydrocarbon chain-melting phase transitions, exhibits no calorimetrically detectable hysteresis [see Lewis *et al.* (1987)].

The incorporation of GS into these lipid vesicles significantly alters the contours of the observed DSC thermograms. Specifically, GS suppresses the pretransition, broadens the main phase transition, and induces the appearance of a high-temperature shoulder on the latter. However, the overall temperature of the main phase transition increases only slightly, and the phase transition enthalpy decreases only slightly (<10%), even at lipid−peptide ratios near 25:1. Thus, although GS clearly interacts with and perturbs the packing of DMPC bilayers, the observed effects on the DMPC thermotropic phase behavior are relatively modest. Moreover, these results differ markedly from the large increases in peak width, and the large decreases in both main phase transition temperature and transition enthalpy, that are normally observed when comparable amounts of transmembrane proteins or peptides are incorporated into lipid bilayers [for examples, see Zhang *et al*. (1992, 1995)]. Instead, the effect of GS on the thermotropic phase behavior of DMPC is compatible with that expected of class II membrane proteins as defined by Papahadjopoulos *et al*. (1975) and McElhaney (1986). Such proteins are localized largely at the bilayer surface and interact primarily with the bilayer headgroup and polar/apolar interfaces. We therefore conclude that GS is not deeply buried in the hydrophobic core of the lipid bilayer. Instead, the peptide interacts primarily with the bilayer surface and interfacial regions by electrostatic and/or hydrogen-bonding interactions, its perturbation of the hydrocarbon chain packing being a secondary effect of such surface interactions. Similar conclusions were reached in a previous study of DPPC−GS interactions (Datema *et al*., 1986).

Figure 1 also shows that both DMPC alone and DMPC−GS (25:1) mixtures exhibit axially symmetric ^{31}P-NMR powder patterns in both the gel and liquid-crystalline states. As is typical of the *n*-saturated PCs, the basal line width of these axially symmetric powder patterns decreases significantly upon melting of the lipid hydrocarbon chains, but the overall contours of the lamellar phase powder pattern do not change substantially, even when heated to temperatures well above the T_m. These results indicate that in both the gel and liquid-crystalline states, DMPC exists exclusively as bilayers in which the phosphate headgroup can undergo relatively fast axially symmetric reorientation, while the assembly itself reorients very slowly on the ^{31}P-NMR time scale (Seelig, 1978). Thus, despite the calorimetrically detectable changes in the thermotropic phase behavior noted above and the relatively high lipid−peptide ratios used here (25:1), the interaction of GS with DMPC dispersions does not cause any major disruption of the bilayer structure or significant changes in the anisotropy of phospholipid headgroup motions. Similar conclusions have been drawn from the results of a previous study (Zidovetzki *et al*., 1988).

The effects of GS on the thermotropic phase behavior of large, multilamellar dispersions of DMPE and DEPE are summarized in Figures 2−4. As illustrated in Figure 2, the gel/liquid-crystalline (L_β/L_α) phase transitions of pure DMPE and DEPE are manifest calorimetrically by single, highly cooperative heating endotherms centered near 50.4 and 37.8 °C, respectively. Upon cooling, the L_α/L_β phase transitions of these lipids are manifest by seemingly multicomponent exotherms at slightly lower temperatures (48.4 and 35.6 °C

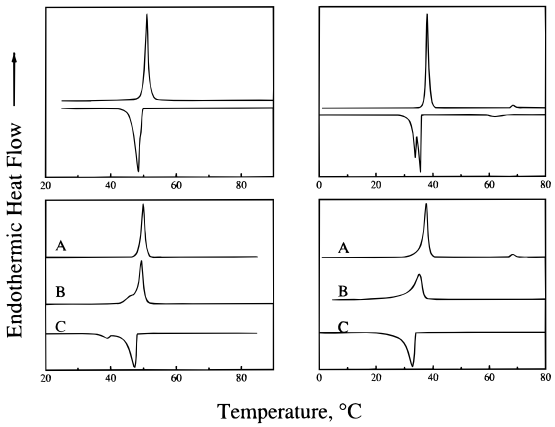

FIGURE 2: DSC thermograms illustrating the effect of GS on the thermotropic phase behavior of DMPE (left panel) and DEPE (right panel) dispersions. Heating and cooling thermograms of the pure lipid species are shown in the top panels. The thermograms shown in the bottom panels were obtained with a lipid−peptide mixture (25:1). (A) Heating thermograms recorded before heating the sample to higher temperatures. (B) Heating thermograms recorded after heating the sample to higher temperatures. (C) Cooling thermogram recorded after heating the sample to higher temperatures.

for DMPE and DEPE, respectively). This pattern of behavior is highly reproducible and has been observed in previous studies of fully hydrated *n*-saturated PE bilayers [see Yao *et al*. (1992) and Lewis and McElhaney (1993)]. In the case of DEPE, a weaker transition centered near 65 °C is also observed. This event is the lamellar liquid-crystalline to inverted hexagonal (L_α/H_{II}) phase transition. The thermotropic phase behaviors of DMPE and DEPE dispersions are not affected by repeated heating and cooling over the temperature range 0−90 °C.

The thermotropic phase behavior of GS−PE mixtures, however, does vary with the thermal history of the sample, specifically by whether or not the samples are heated to high temperatures. Thus, for mixtures of gramicidin S with both DMPE and DEPE, the DSC thermograms are slightly broader, but the temperature and enthalpy of the chain-melting phase transition are almost identical to those exhibited by the pure lipid, as long as the lipid−peptide samples are not exposed to high temperatures ($> T_m + 15$ °C) (see thermograms A in Figure 2). However, the DSC thermograms change irreversibly after the lipid−peptide samples have been heated to higher temperatures (see thermograms B and C in Figure 2). Once exposed to higher temperatures, succeeding DSC heating thermograms indicate that the lipid L_β/L_α phase transitions appear as superpositions of broad and narrow components, both of which are centered at temperatures below that of the pure lipid species. Specifically, the sharp component is centered at temperatures just below that of the pure lipid, whereas the broader component is centered at temperatures some 5−7 °C lower. Also, in the case of DEPE, the L_α/H_{II} phase transition is not calorimetrically detectable after the sample has been heated to higher temperatures (see thermograms B and C in Figure 2). These results indicate that the PE−GS interactions which occur when the samples are initially dispersed at modest temperatures are less extensive than those which occur after samples have been heated to high temperatures, possibly because GS can interact more strongly with more fluid bilayers. The basis of this difference in thermotropic phase behavior was examined further by ^{31}P-NMR spectroscopy.

The ^{31}P-NMR spectra shown in the left panel of Figure 3

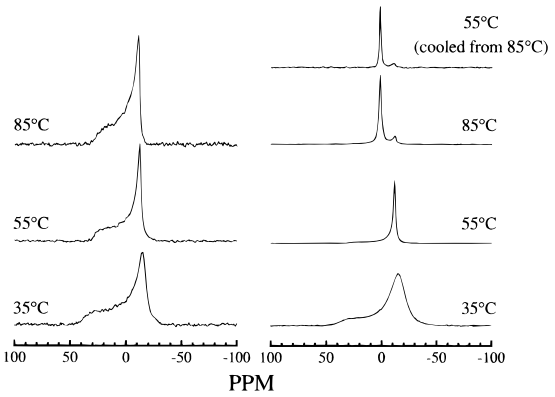

FIGURE 3: Proton-decoupled ^{31}P-NMR spectra of DMPE (left panel) and of a 25:1 mixture of DMPE with GS (right panel). Unless otherwise stated, spectra were acquired in the heating mode at the temperatures indicated. The spectra shown in the right panel were acquired after initially heating the sample to temperatures near 90 °C.

typify those obtained in the L_β ($T < 50$ °C) and L_α ($T > 50$ °C) states of DMPE alone throughout the temperature range studied. These spectra are typical of those normally exhibited by gel and liquid-crystalline bilayers of most of the common phospholipids. For the most part, the spectra exhibited by the pure lipid (Figure 3, left panel) are similar to those exhibited by the gel and liquid-crystalline states of DMPE−GS mixtures that have not been heated to temperatures above 65 °C. Indeed, the only differences observed are the slightly smaller basal line widths and the less prominent downfield shoulders of the powder pattern spectra of the peptide-containing as compared to the pure lipid samples. These differences in the ^{31}P-NMR spectra can be rationalized two ways. First, the intensities of various regions of such ^{31}P-NMR powder patterns may be dependent on the angular dependence of the transverse and longitudinal components to the relaxation of the ^{31}P nuclei in the magnetic field [see Hemminga and Cullis (1982)]. Thus, it is possible that GS binding may be affecting the angular dependence of the ^{31}P relaxation in these phospholipid bilayers. Second, the results of previous studies of phospholipid model membranes in which qualitatively similar ^{31}P-NMR spectra have been observed [for examples, see Seelig *et al*. (1985), Speyer *et al.* (1987), Bitbol *et al.* (1989), Jansson *et al.* (1990), Sanders and Prestegard (1990), and Qiu *et al.* (1993)] have been ascribed to magnetically induced sample orientation, especially in the liquid-crystalline states of such lipid dispersions (Speyer *et al*., 1987; Qiu *et al.*, 1993). Furthermore, it has been suggested that the susceptibility of lipid membranes to orientation in an applied magnetic field may be related to their curvature and elastic properties (Speyer *et al*., 1987). If such is the case here, then our data suggest that the changes in the curvature and/or elastic properties of PE bilayers coincident with their interaction with gramicidin S markedly increase the probability of their spontaneous alignment in an applied magnetic field. We favor the latter possibility.

Figure 3 (right panel) also shows that the contours of the ^{31}P-NMR powder patterns change dramatically when the DMPE−GS sample is heated to temperatures above 70 °C. Initially, the NMR spectrum appears to be a superposition of the axially symmetric powder pattern described above and a sharp isotropic component centered near 2 ppm downfield. The latter grows in intensity with increases in temperature, and, at temperatures near 85 °C, it is the dominant feature

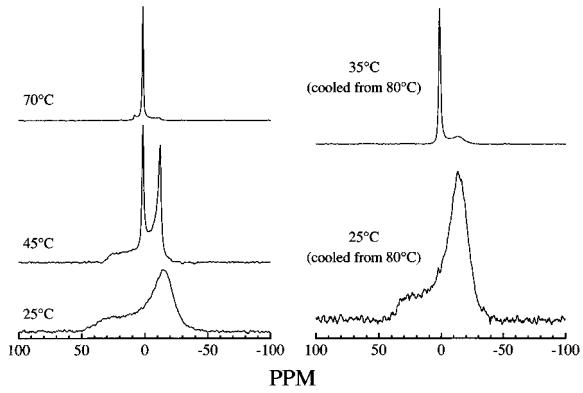

FIGURE 4: Proton-decoupled ^{31}P-NMR spectra of a 25:1 mixture of DEPE with GS. Spectra were obtained using a sample that was dispersed at temperatures near 50 °C prior to initial data acquisition. Unless otherwise stated, spectra were acquired in the heating mode.

of the NMR spectrum. Clearly, a marked change in the organization of the gramicidin S-containing DMPE dispersions occurs when these lipid−peptide mixtures are taken to high temperatures. Interestingly, the spectroscopic changes observed at high temperatures are not freely reversible upon cooling. As illustrated in Figure 3 (right panel), the structure which gives rise to the sharp isotropic component remains stable even when the lipid−peptide sample is cooled to temperatures well below those at which the isotropic component first appears on heating. In fact, the isotropic component disappears only when the samples are cooled to temperatures well below the T_m of the pure lipid. We also find that after the lipid−peptide sample has been heated to higher temperatures and recooled to temperatures well below T_m, the contours of the axially symmetric powder patterns observed in the L_β and in the L_α states are not the same as those observed previously. Specifically, the relative intensities of the downfield components of the powder patterns observed in these L_β and L_α bilayers are significantly diminished when compared with those acquired prior to heating the samples to high temperature (see Figure 3, right panel). This overall pattern of behavior has been observed in all of the PE−gramicidin S mixtures that we have studied.

Our studies of DEPE−GS mixtures also revealed another interesting aspect of the effect of GS incorporation on PE bilayers. Unlike the DMPE−GS mixture, we find that the isotropic component in the ^{31}P-NMR spectra of the DEPE−peptide mixture is observed at temperatures relatively close to T_m (see Figure 4, left panel). The relative intensity of this isotropic component steadily grows as the temperature increases, and it becomes the dominant feature of the ^{31}P-NMR spectrum as the temperature approaches the lipid L_α/H_{II} phase transition temperature. At this temperature, the residual axially symmetric powder pattern abruptly converts to that typical of the H_{II} phase, and the contours of the overall spectrum resemble that expected of a mixture of isotropic and H_{II} phases (see Figure 4, left panel). Further heating of the sample does not result in any significant changes in the contours of the ^{31}P-NMR spectrum. Upon cooling, the H_{II} phase component of the spectrum reverts to that typical of a lamellar L_α phase at temperatures just below the L_α/H_{II} phase transition temperature of the pure lipid. However, the isotropic component persists to temperatures just below the L_α/L_β phase transition temperature of the mixture (Figure 4, right panel). Total reversion to features characteristic of the lamellar phase occurs only when the sample is cooled to temperatures near or below 25 °C. Subsequent reheating of the sample to high temperatures results in a progressive decline in the relative intensity of the H_{II} phase component that is observed at high temperature. These results indicate that after the DEPE−GS mixture is taken to high temperatures, the formation of the structure(s) giving rise to the isotropic ^{31}P-NMR signal is favored in subsequent heatings over the formation of the H_{II} phase. This observation is consistent with our calorimetric studies, which indicate that the L_α/H_{II} phase transition endotherm is suppressed after the DEPE−GS mixture is heated to high temperatures.

The appearance of the isotropic component in the ^{31}P-NMR spectrum of the PE−GS mixtures is particularly interesting given the results of a recent study demonstrating that alamethicin, a membrane-disrupting peptide, can induce the formation of a cubic phase in aqueous PE dispersions, even at very high lipid−peptide ratios (Keller *et al.*, 1996). Indeed, the possibility that GS may induce the formation of a cubic phase is consistent with the appearance of the isotropic component in our ^{31}P-NMR spectra (Gruner *et al.*, 1985; Tilcock *et al.*, 1986), and with the persistence of this isotropic component at temperatures well below those at which it is initially observed upon heating (Shyamsunder *et al.*, 1988; Veiro *et al.*, 1990; Lewis *et al.*, 1994). However, because the appearance of isotropic peaks in the ^{31}P-NMR spectra of aqueous phospholipid dispersions is not a unique indicator of cubic phase formation (Thayer & Kholer, 1981; Tilcock *et al.*, 1986), X-ray diffraction studies were performed in order to obtain additional information about the structural basis of the isotropic component observed here.

Figure 5 shows representative X-ray powder diffraction patterns exhibited by a DMPE−GS mixture at temperatures just above and well above T_m. At temperatures near 60 °C (T_m + 10 °C), first- and second-order reflections consistent with a lamellar repeat spacing of some 53.8 Å are observed (Figure 5A). This pattern of reflections persists over the entire temperature range of our experiments, and, as expected of liquid-crystalline lipid bilayers, the lamellar repeat spacings decrease modestly with increases in temperature (to 46.4 Å at 95 °C). At elevated temperatures (≥85 °C), additional weaker reflections appear in the diffraction pattern (Figure 5B). The appearance of these additional reflections is consistent with our ^{31}P-NMR spectroscopic evidence for the emergence of a new phase coexisting with the lamellar phase at these elevated temperatures. The additional reflections cannot be indexed on the coexisting lamellar lattice, nor have we been able to identify any single lattice which fits all of the additional reflections. At these elevated temperatures, the two-dimensional diffraction patterns also exhibit a collection of smooth and spotty rings (see Figure 5C), the former of which are located at positions expected of a lamellar phase with a long spacing of 46.4 Å. To our knowledge, the observed pattern of spotty rings has never been observed with either lamellar or H_{II} lipid phases, but is commonly observed with three dimensionally ordered phases such as bicontinuous cubic phases. In Figure 5C, most of these reflections are shown relative to the expected positions of two cubic lattices of the gyroid type. However, this should not be taken as definitive evidence for the existence of gyroid lattices because there are too few reflections to make such an assignment unequivocal. Nevertheless, the combination of X-ray diffraction and ^{31}P-NMR spectroscopic data acquired is consistent with the existence of polydomain samples in which a lamellar phase coexists with other three dimensionally ordered nonlamellar phases

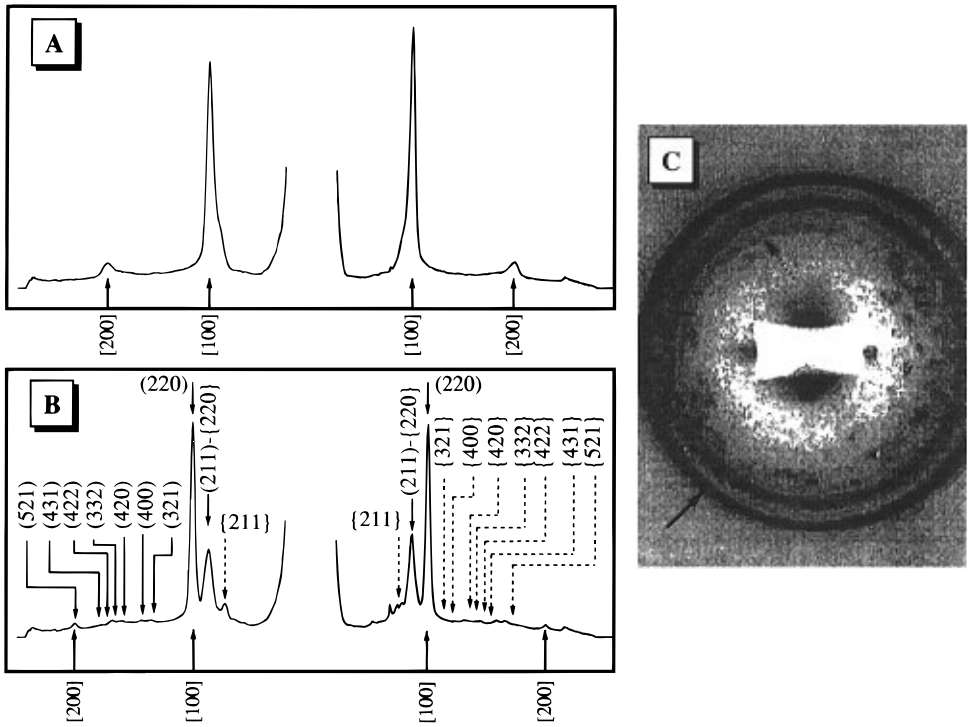

FIGURE 5: X-ray diffraction patterns exhibited by DMPE−GS mixtures (25:1). Panels A and B show azimuthally integrated patterns acquired at 60 and 95 °C, respectively. The reflections marked in panel A are the first [100] and second [200] order reflections of a lamellar phase with a basis of 53.8 Å. In panel B, the brackets indicate the first- and second-order reflections of a lamellar phase with a basis of 46.4 Å, the parentheses indicate the positions of reflections expected of a gyriod lattice with a basis of 131.4 Å, and the braces indicate the positions of reflections expected of a gyriod lattice with a basis of 152.8 Å. Panel C shows the two-dimensional diffraction pattern used to derive the integration profile shown in panel B. Note the combination spotty rings (dashed arrow) indicative of three dimensionally periodic lattices and coexisting smoother rings (solid arrow).

(probably inverted cubic phases) at elevated temperatures. That GS actually induces the formation of such structures in a DMPE matrix seems remarkable, considering that hydrated DMPE is not known to form type II phases near 90 °C, nor is it expected to do so at temperatures below 150−160 °C [see Lewis *et al*. (1989)]. Our data therefore suggest that GS has considerable potential for destabilizing PE bilayers with respect to highly curved inverted nonlamellar phases.

^{31}P-NMR spectroscopic studies of the effect of GS on DPEPE dispersions were also performed to gain additional insight into the temperature and peptide concentration dependence of the capacity of GS to induce the formation of cubic or other three dimensionally ordered phases in PEs. DPEPE is well suited for this type of study because of its low L_β/L_α phase transition temperature and relatively high L_α/H_{II} phase transition temperature [20.7 and 92.5 °C, respectively, see Silvius *et al*. (1985)]. It was thus feasible for us to form liquid-crystalline lipid and lipid−peptide dispersions at relatively low temperatures (∼35 °C) while avoiding a close approach to the lipid lamellar/nonlamellar phase transition temperature. The data shown in Figure 6 were obtained from pure DPEPE and DPEPE−GS mixtures that were dispersed at 35 °C and recooled to 25 °C (∼5 °C above the T_m) prior to data acquisition. It is clear that pure DPEPE retains a lamellar structure to at least 70 °C. As expected, the interaction of GS with this lipid results in the appearance of isotropic components in the ^{31}P-NMR spectrum at temperatures well below that of the normal lamellar/nonlamellar phase transition of the lipid (∼90 °C under our conditions). Also, it is clear that the temperature at which the isotropic component first appears decreases significantly with increases in the peptide concentration. Thus, at lipid:

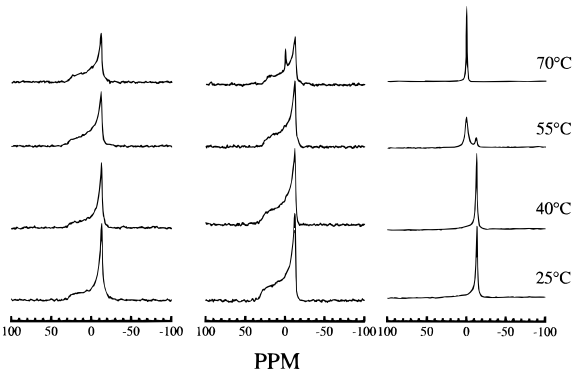

FIGURE 6: Temperature dependence of the ^{31}P-NMR spectra of 1,2-dipalmitelaidoyl-PE and its mixtures with GS. Spectra of the pure lipid are shown in the left panel, and those of the lipid−peptide mixtures (100:1 and 25:1) are shown in the middle and right panels, respectively. All data were acquired in the heating mode with samples that were dispersed at temperatures near 35 °C.

GS ratios near 100:1, the isotropic component is first observed at temperatures near 70 °C, whereas at lipid:peptide ratios near 25:1, it is first observed in the 45−55 °C temperature range. It is important to note, however, that the data shown in Figure 6 were obtained with samples that never approached the lipid lamellar/nonlamellar phase transition temperature range prior to data acquisition. Previous studies have shown that long-lived cubic phases are often formed when PEs are cycled through temperatures bracketing the L_α/H_{II} phase transition (Shyamsunder *et al*., 1988; Veiro *et al*., 1990). The seemingly stable cubic phases formed under such conditions are believed to be metastable structures which have been kinetically trapped by rapid cycling through the L_α/H_{II} phase transition (Shyamsunder *et al*., 1988). The GS-induced cubic phase formation which we observe at

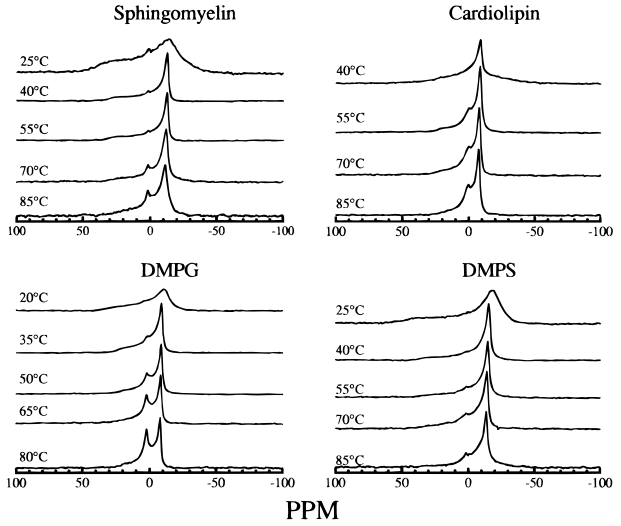

FIGURE 7: Proton-decoupled ^{31}P-NMR spectra exhibited by mixtures of GS with various phospholipids. The spectra shown were all acquired in the heating mode at the temperatures indicated, and at a lipid:peptide molar ratio of 25:1.

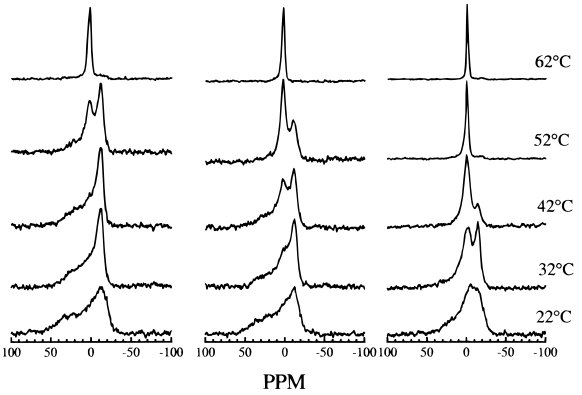

FIGURE 8: Temperature dependence of the ^{31}P-NMR spectra of 1,2-di-(DL-2-butylhexadecanoyl)-PC and its mixtures with gramicidin S. Spectra of the pure lipid are shown in the left panel, and those of the lipid−peptide mixtures (100:1 and 25:1) are shown in the middle and right panels, respectively. All data were acquired in the heating mode from samples that were initially dispersed at temperatures near 30 °C and reequilibriated at 0−4 °C prior to data acquisition.

temperatures well below the lipid L_α/H_{II} phase transition temperature is thus not the manifestation of similar phenomena. Instead, our results appear to be a reflection of a thermodynamically driven increase in lipid nonlamellar phase-forming propensity induced by GS.

^{31}P-NMR spectroscopic studies of mixtures of GS with a number of other phospholipid classes were performed to determine whether the peptide could also induce the formation of cubic or other three dimensionally ordered phases in lipid systems other than PE. Aside from the PC- and PE-based lipid−peptide mixtures described above, we have examined mixtures of GS with DMPG, DMPS, TMCL, and brain SpM at temperatures up to 85−90 °C (see Figure 7). The capacity of GS to induce the formation of such phases seems to be very limited with all of the other lipid classes examined with the probable exception of DMPG. With all of the lipids studied, small isotropic peaks are present in their ^{31}P-NMR spectra, suggesting the possibility of cubic phase formation. However, this seems unlikely with the mixtures of GS with DMPS, TMCL, and SpM because the relative intensities of these small isotropic peaks do not change significantly with temperature, suggesting that they arise from a minor population of small, freely tumbling vesicles. With the PG−GS mixture, however, the relative size of the isotropic component of the ^{31}P-NMR spectrum is significantly greater than that observed with all lipid−peptide mixtures except for the PE-based systems already described; the relative size of this component increases with temperature (see Figure 7), and it exhibits considerable hysteresis upon cooling (data not shown). A similar pattern of results was observed in our studies of POPG−GS mixtures (data not shown). These observations are consistent with the possibility that upon interaction with PG, GS may actually induce the formation of nonlamellar phases similar to those identified in our X-ray diffraction studies. However, we were unable to unequivocally prove or disprove this suggestion, because the population of PG molecules which give rise to the isotropic component of the spectrum constitutes a relatively small fraction of the total phospholipid population. This fact makes it difficult to use X-ray diffraction to address this issue because, as is evident from the data shown in Figure 5, resolution of the weak reflections arising from such three dimensionally ordered phases, in the presence of the intrinsically stronger reflections from the lamellar phase, is difficult in a mixed- phase system, even when the former is the dominant component in the mixture. Nevertheless, it does appear that the effect of GS on lipid phase behavior is strongly dependent upon the structure of the lipid polar headgroup. This observation has interesting implications for the possible mechanism of the membrane disruption by this peptide which will be examined further in the Discussion.

The ^{31}P-NMR spectroscopic data presented above indicate that GS-induced nonlamellar phase formation is more effective with PEs than with any of these other phospholipid classes studied here. Since PEs are intrinsically more prone to form nonlamellar phases than are any of the other phospholipids classes [see Lewis *et al*. (1997)], the question arises as to whether the observed peptide-induced nonlamellar phase formation is a reflection of the specificity of interactions between GS and the PE headgroup or of the intrinsically high nonlamellar phase-forming tendencies of PE. This possibility was explored by an examination of the effects of GS on 1,2-di-(DL-2-butylhexadecanoyl)-PC, a novel nonlamellar phase-forming PC that was recently developed in this laboratory [see Lewis *et al.* (1994)]. This particular lipid is well suited for this study because it has a relatively low T_m (∼26 °C) and a reasonable temperature gap between the gel/liquid-crystalline phase transition and the onset of the formation of nonlamellar structures. Our results indicate that the pure lipid exhibits axially symmetric ^{31}P-NMR powder patterns at all temperatures below 40−45 °C, that isotropic components first emerge in its ^{31}P-NMR spectra at temperatures near 50 °C, and that conversion of the axially symmetric powder patterns to an isotropic signal is virtually complete at temperatures above 65 °C (see Figure 8). These observations are compatible with the results of previously published work, in which a correlation between the appearance of the isotropic signal in the ^{31}P-NMR spectrum and the conversion from a lamellar phase to a cubic phase was established [see Lewis *et al*. (1994)]. It is also clear from Figure 8 that the addition of GS to this PC dramatically reduces the temperature over which conversion from a lamellar phase to a cubic phase occurs. For example, at lipid:peptide ratios near 100:1, evidence for cubic phase formation first emerges at temperatures near 35 °C, and the process is

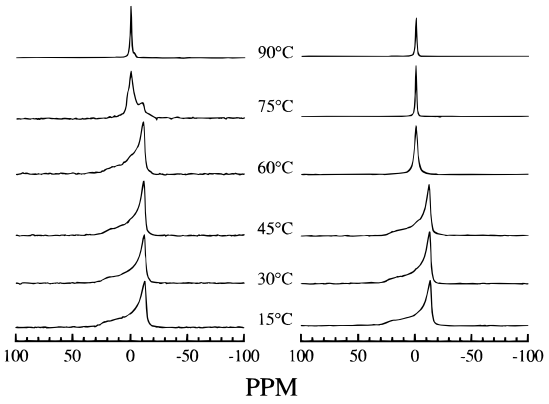

FIGURE 9: Proton-decoupled ^{31}P-NMR spectra of an aqueous dispersion of *Escherichia coli* membrane polar lipids (left) and a 25:1 mixture of these lipids with GS (right panel). The samples were dispersed at temperatures near 35 °C and spectra were acquired in the heating mode at the temperatures indicated.

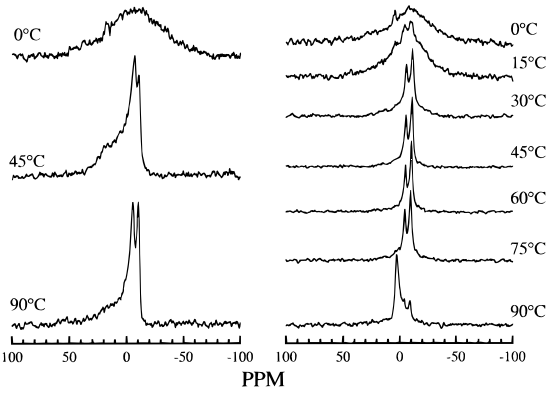

FIGURE 10: Proton-decoupled ^{31}P-NMR spectra of an aqueous dispersion of *Acholeplasma laidlawii* B membrane lipids (left) and a 25:1 mixture of these lipids with GS (right panel). The spectra were acquired in the heating mode at the temperatures indicated.

essentially complete at temperatures near 55 °C. At lipid−peptide ratios near 25:1, however, evidence for cubic phase formation is observed at all temperatures above T_m, and conversion to the cubic phase is virtually complete at temperatures near 50 °C. These results contrast sharply with those obtained with the DMPC−GS mixtures (see above) and clearly indicate that GS can induce the formation of cubic phases when mixed with lipids other than PE. Indeed, these results suggest that the capacity of GS to induce the formation of cubic or other nonlamellar phases when mixed with various phospholipids is linked to the intrinsic nonlamellar phase-forming propensities of the lipids themselves rather than to particular lipid polar headgroup structures.

Finally, the effects of GS on some microbial membrane polar lipid dispersions were examined to determine whether this peptide can induce or potentiate nonlamellar phase formation in model membranes derived from the heterogeneous lipid mixtures which occur in natural cell membranes. Polar lipid extracts from the membranes of *E. coli* and *A. laidlawii* B were used for this study, and the results are summarized in Figures 9 and 10. Our results indicate that at temperatures between 15 and 60 °C, *E. coli* polar lipid extracts exhibit axially symmetric ^{31}P-NMR powder patterns typical of the lamellar liquid-crystalline state phospholipid bilayers (see Figure 9, left panel). At temperatures above 65−70 °C, isotropic components first appear in the ^{31}P-NMR spectra, and the relative intensity of these components increases significantly with further increases in temperature (Figure 9, left panel). These observations indicate that the lamellar liquid-crystalline phase of this lipid mixture begins conversion to a nonlamellar phase at temperatures between 65 and 70 °C and that this process is essentially complete at temperatures near 90 °C. With the corresponding lipid−GS mixture (25:1), axially symmetric ^{31}P-NMR powder patterns are also observed at temperatures up to 45 °C (Figure 9, right panel). However, unlike the corresponding lipid sample without GS, conversion to a nonlamellar phase begins at temperatures near 50 °C, and, as illustrated in Figure 9 (right panel), the process is essentially complete at 60 °C. This result clearly demonstrates that the presence of GS significantly reduces the temperature at which the *E. coli* polar lipids form nonlamellar phases.

The corresponding studies of *A. laidlawii* B polar lipid extracts yielded results which are generally similar to those described above (see Figure 10). At gel-state temperatures (∼0 °C), the polar lipid extract exhibits very broad ^{31}P-NMR powder patterns which are indicative of relatively slow motions of the ^{31}P nuclei on the ^{31}P-NMR time scale (see Figure 10, left panel). The signals originate from the membrane phospholipids PG and the phosphorylated glycolipid GPDGDG [for more details of the overall lipid composition of these membranes and the chemical structure of GPDGDG, see Smith (1992) and Monck *et al*. (1992)]. When heated to temperatures above the T_m (>25 °C), the ^{31}P-NMR spectrum assumes the contours of a predominantly axially symmetric powder pattern over which is superimposed a relatively small sharp peak centered 6 ppm upfield (see Figure 10). The occurrence of the latter can be attributed to the fact that the chemical shielding anisotropy of GPDGDG differs from that of common phospholipids such as PG (Lindblom *et al.*, 1986). With the sample shown in Figure 10 (left panel), axially symmetric ^{31}P-NMR powder patterns are retained at temperatures near 90 °C, indicating that the lamellar phase is the thermodynamically preferred phase at those temperatures. However, we have also observed that at temperatures near 90 °C, the ^{31}P-NMR spectra of comparable samples of *A. laidlawii* polar lipid extracts may also exhibit a small sharp peak near 2 ppm downfield as a relatively minor component of the powder pattern (data not shown). Thus, although the lamellar phase is thermodynamically preferred over nonlamellar phases at temperatures near 90 °C, this temperature range is probably very close to the lower end of the range at which the polar lipid extracts of these *A. laidlawii* B membranes will begin to convert to a nonlamellar phase. Our results also show that ^{31}P-NMR spectra of the mixture of *A. laidlawii* polar lipid extract with GS (25:1) differ significantly from those exhibited by the lipid extract alone at all temperatures above the T_m. Specifically, the upfield components of the ^{31}P-NMR powder patterns are considerably sharper than observed in the absence of the peptide, and the relative intensities of the downfield components of the powder pattern are smaller than is observed with the polar lipid extract alone (see Figure 10). This observation is generally compatible with the effects of GS on the ^{31}P-NMR spectra of all of the phospholipids studied here. We also note that at temperatures near 90 °C, the dominant feature of the ^{31}P-NMR spectrum of the lipid−GS mixture is a sharp peak centered near 2 ppm downfield (see Figure 10, right panel). The appearance of the latter indicates that substantial conversion to a nonlamellar phase occurs at temperatures near 90 °C, in marked contrast to what is observed with the lipids alone. Clearly, as was observed

with the *E. coli* polar lipids, interaction of GS with lipid bilayers derived from the polar lipids of *A. laidlawii* B membranes results in a significant lowering of the temperature over which the lamellar to nonlamellar phase transition occurs. It should also be noted that unlike *E. coli* and mammalian membranes, glycolipids are the predominant structural components of the *A. laidlawii* B membrane. The demonstration that GS can potentiate the formation of nonlamellar phases in glycolipid-based membranes indicates that this property is not limited to phospholipid bilayers.

DISCUSSION

A major feature of this work is the demonstration that GS can induce the formation of three dimensionally ordered nonlamellar structures (most probably inverted cubic phases) when mixed with certain types of lipids. At lipid:peptide ratios near 25:1, we find strong evidence for nonlamellar phase formation when GS is incorporated into bilayers composed of one of several PEs, a 2-alkyl-substituted PC, and polar lipid extracts of *A. laidlawii* and *E. coli* membranes and, to a smaller extent, of DMPG. However, under the same conditions we find little evidence for nonlamellar phase formation when GS is mixed with either DMPC, DMPS, brain SpM, or TMCL. Thus, the capacity of GS to induce nonlamellar phase formation seems to be lipid polar headgroup dependent. However, one should note that previous workers have observed a pronounced isotropic component in the ^{31}P-NMR spectra of DMPC−GS mixtures at very high peptide contents (Zidovetski *et al.*, 1988). These earlier results are compatible with our own experiments showing isotropic components in the ^{31}P-NMR spectra of DMPC−GS mixtures at temperatures above 70 °C when a very low (10:1) lipid:GS ratio is used (data not shown). The isotropic spectral component reported by Zidovetski and co-workers was only observed at temperatures above the lipid T_m, and it exhibited considerable cooling hysteresis, reverting to the typical lamellar phase powder pattern only when cooled to temperatures well below T_m. Such behavior is typical of phospholipid cubic phases generally [see Lewis *et al.* (1994)] and is strikingly similar to what we observed here in our PE−GS mixtures. It is therefore possible that Zidovetski *et al.* (1988) were actually observing the formation of nonlamellar phases in their DMPC−GS mixtures and not populations of rapidly tumbling lipid−peptide micelles, as they have assumed. Thus, GS will probably induce nonlamellar phases in most lipids if sufficiently high levels of peptide are incorporated. We therefore suggest that the lipid specificity noted in our study is a manifestation of quantitative rather than qualitative differences in the susceptibility of the various lipids to GS-induced nonlamellar phase formation. Most probably, this phospholipid specificity will be even more pronounced at lower and more physiologically relevant peptide concentrations.

In principle, the lipid specificity of the GS-induced effects on lipid polymorphism observed here can be rationalized by considering the intrinsic nonlamellar phase-forming tendencies of the lipids examined. At lipid:peptide ratios near 25:1, lipids which exhibit a high propensity to form nonlamellar phases (the PEs and the 2-alkyl chain-substituted PC) and membrane lipid dispersions containing significant amounts of such lipids (PE for *E. coli* lipids; monoglucosyl diacylglycerol for *A. laidlawii* lipids), are induced to form nonlamellar phases at much lower temperatures than observed in the absence of GS. Moreover, with the PEs a positive correlation is observed between GS-induced nonlamellar phase formation and their intrinsic nonlamellar phase-forming tendencies (i.e., DPEPE > DEPE > DMPE). In contrast, lamellar phase-preferring lipids such as DMPC, DMPS, brain SpM, and TMCL do not form nonlamellar phases in the presence of GS under comparable conditions. The one exception to this general rule appears to be DMPG. This lipid is not known to form nonlamellar phases under physiologically relevant conditions but appears to exhibit such properties when mixed with GS. The reasons for this apparent anomaly are currently unclear. It is interesting to note, however, that although the incorporation of small amounts of PG into a PE matrix significantly broadens the PE lamellar/nonlamellar phase transition, it results in a much smaller net elevation of the lamellar/nonlamellar phase transition temperature than do comparable amounts of other bilayer-preferring lipids such as PC and PS (Foht *et al.*, 1995). Moreover, in contrast to PC and PS, PG also induces the formation of a cubic phase in the PE host bilayer (Foht *et al.*, 1995). Thus, although PGs are normally considered to be lamellar-preferring lipids, they may actually have a greater intrinsic propensity for forming nonlamellar phases than the other nominally bilayer-preferring lipids examined here.

Although the intrinsic inverted nonlamellar phase-forming propensity of the target lipid bilayer seems to be the major factor in determining the specificity of the effect on GS of lipid polymorphic phase behavior, we do not wish to imply that other factors are unimportant for the interaction of this peptide with lipid model or biological membranes. For example, the stronger interaction of GS with PC as compared to PE bilayers at comparable reduced temperatures, and the stronger interaction of GS with PE bilayers at higher as compared to lower temperatures, as observed calorimetrically, suggests that the strength of phospholipid−peptide interactions may also be positively correlated with the fluidity of the lipid bilayer in the liquid-crystalline state. This suggestion is supported by the fact that the addition of cholesterol to fluid phospholipid bilayers decreases the magnitude of the effect of GS addition on lipid thermotropic phase behavior generally (unpublished observations from this laboratory). Additional studies using other physical techniques will be required to determine if other, more subtle properties of the various lipid polar headgroups significantly influence the binding and partial penetration of GS into phospholipid bilayers, and the nature of the interaction of this peptide with various phospholipid molecules.

Our finding that, in general, GS does not appear to interact particularly strongly with the anionic phospholipids tested is perhaps surprising, given that the presence of positively charged residues in the GS molecule seems to be required for its antimicrobial effects, and that electrostatic interactions between GS molecules and their presumably negatively charged target molecules are important for the cell-killing effects of this peptide. However, since anionic lipids in general are less prone to form inverted nonlamellar phases, this effect may be masking the effects of favorable electrostatic interactions in the present study, where the ability of GS to induce the formation of inverted cubic phases is the primary focus. Therefore, we do not mean to imply that electrostatic interactions between the basic amino acid residues of GS and anionic phospholipids may not be important *in vivo*, for example by increasing the rate and/or extent of peptide binding to the bilayer surface, thus

facilitating the interaction of GS with lipid components capable of forming localized regions of nonlamellar structure. Moreover, since even the zwitterionic phospholipids possess a negatively charged phosphate moiety at physiological pHs, electrostatic interactions between GS molecules and this portion of the lipid polar headgroup may still be possible, despite like-charge repulsive effects from the positively charged nitrogen atom of the polar headgroup. Nevertheless, our results do not support the postulate that the presence of anionic phospholipids is required for GS binding to, or destabilization of, the lipid bilayers of target membranes.

The possibility that the membrane-disrupting capacity of GS is related to the nonlamellar phase-forming propensity of the lipids of the target membrane has important implications for our understanding of the mechanism of action and the specificity of this antimicrobial peptide. Current ideas suggest that the nonlamellar phase-forming propensities of lipid bilayers are largely determined by their monolayer curvature stress (Kirk *et al.*, 1984; Gruner, 1985). This stress arises naturally from the fact that in each leaflet of a lipid bilayer, forces arising from interactions in the polar, the interfacial, and the hydrophobic regions do not act through the same "center of mass". Because of this, the vector sum of the moments of these forces about the center of mass of the lipid molecules is not zero, and, as a result, each of the monolayers will be predisposed to curvature. In liquid-crystalline bilayers, these spontaneous curvature tendencies are opposed by the packing requirements of the lipid hydrocarbon chains, and, as a result, the bilayer becomes highly stressed, because both the curvature and the hydrocarbon chain-packing tendencies toward their own free energy minima are simultaneously frustrated [for more detailed discussions of these concepts, see Helfrich (1973), Kirk *et al.* (1984), and Gruner (1985, 1982)]. Given that GS promotes the formation of highly curved structures such as cubic phases when it interacts with lipid bilayers, we can conclude that GS binding to lipid bilayers increases the monolayer curvature stress. Also, because membrane-bound peptide molecules are likely to perturb only those lipid molecules in their immediate vicinity, the increase in monolayer curvature stress may also be highly localized. We therefore suggest that the occurrence of localized domains of high curvature stress may be key to the membrane lytic activities of GS, since defects occurring at the boundaries of these domains may function as sites through which the leakage of cellular contents may occur. Moreover, because of the highly localized effects of such lipid−peptide interactions, these defects can begin to accumulate at very low peptide:lipid ratios. As well, the number of such defects will increase with increases in the intrinsic monolayer curvature stress of the target membrane, or when large disparities between the curvature stress of peptide-rich and peptide-poor membrane domains exist. Given this, one can envisage how the degradation of membrane barrier properties and other forms of membrane destabilization can readily occur once the number of such defects exceeds some critical value. Whether the defects which exist at physiologically relevant concentrations of GS are due to the actual formation of nonlamellar lipid phases, or to other discontinuities in the structure of the lipid bilayer of the target membrane, remains to be determined.

It is clear that GS can disrupt the structural integrity of lipid bilayer membranes by promoting the formation of inverted (i.e., type II) nonlamellar lipid phases. Although this is the first such demonstration for this particular class of antimicrobial peptides, other peptide antibiotics are known to exhibit similar activities. For example, the linear peptides, such as gramicidins A, B, and C (Killian & de Kruijff, 1986; Killian *et al.*, 1987; Tournois *et al.*, 1990) and alamethicin (Keller *et al.*, 1996), also induce nonlamellar phase formation when incorporated into appropriate lipid dispersions. We therefore suggest that membrane disruption mediated by localized increases in membrane monolayer curvature stress could be a general mode of action of many antimicrobial peptides. For GS in particular, this suggestion is consistent with the observation that this peptide induces the release of small phospholipid particles from *E. coli* and human erythrocyte membranes, a process which seems to be correlated with the breakdown of membrane barrier properties and, ultimately, cell death (Katsu *et al.*, 1986, 1988, 1989). This hypothesis is also supported by the observation that GS induces marked alterations in the shape of erythrocyte membranes (Katsu *et al., 1988*), as do other agents which increase monolayer curvature stress in lipid bilayers. It is also interesting to note that GS exhibits fusogenic properties, since membrane fusion is believed to proceed via inverted nonlamellar lipid phase intermediates (Legendre & Szoka, 1993; Legendre & Supersaxo, 1995; Hara *et al.*, 1996). If our suggestion is correct, then GS at sufficiently high concentrations will probably disrupt virtually all biological membranes, since significant amounts of nonlamellar phase-forming lipids of some type are found in surface membranes from all classes of organisms (Lewis *et al.*, 1997). However, at lower GS concentrations, the susceptibility of biological membranes from different organisms to peptide attack should vary both with the amount of nonlamellar phase-preferring lipid present and with the intrinsic propensity of that particular lipid to form nonbilayer phases. Moreover, it may also be possible to design GS analogues with increased specificity for interactions with the type of nonlamellar phase-preferring lipids found in bacterial but not in mammalian surface membranes. In this way, the antimicrobial activity of this class of peptides could be increased while its cytolytic activity is decreased, thus resulting in a much enhanced therapeutic index. We are actively investigating this possibility.

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